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RESEARCH PAPERS

Characterization of a new partitivirus strain in *Verticillium dahliae* provides further evidence of the spread of the highly virulent defoliating pathotype through new introductions

Mari Carmen CAÑIZARES[1], Encarnación PÉREZ-ARTÉS[2], Nicolás Emilio GARCÍA-PEDRAJAS[3] and María Dolores GARCÍA-PEDRAJAS[1]

[1] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga, Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Estación Experimental "La Mayora", 29750 Algarrobo-Costa, Málaga, Spain
[2] Department of Crop Protection, Instituto de Agricultura Sostenible, IAS-CSIC, Alameda del Obispo s/n. Apdo 4084, 14080 Córdoba, Spain
[3] Department of Computing and Numerical Analysis, C2 Building 3rd Floor, Campus Universitario de Rabanales, 14071 Córdoba, Spain

Summary. The soilborne pathogen *Verticillium dahliae*, causal agent of Verticillium wilt, has a worldwide distribution and many hosts of agronomic value. The worldwide spread of a highly virulent defoliating (D) pathotype has greatly increased the threat posed by *V. dahliae* in olive trees. For effective disease management, it is important to know if the D pathotype is spreading long distances from contaminated material, or if D pathotype isolates may have originated locally from native *V. dahliae* populations several times. We identified a double-stranded RNA mycovirus in an olive D pathotype isolate from Turkey. Sequencing and phylogenetic analysis clustered the virus with members of the family *Partitiviridae*. The virus was most similar to a partitivirus previously identified in a *V. dahliae* isolate from cotton in China (VdPV1), with sequence identities of 94% and 91% at the nucleotide level for RNA1 and RNA2, respectively. The virus therefore corresponded to a strain of the established species, and we designated it VdPV1-ol (VdPV1 from olive). The identification of the same viral species in these two fungal isolates from geographically distant origins provides evidence of their relationships, supporting the hypothesis of long-distance movement of *V. dahliae* isolates.

Key words: Vascular pathogens, defoliating pathotype, migration, mycoviruses, olive trees.

Introduction

Verticillium dahliae is a cosmopolitan soil fungus, which causes vascular wilts in a wide variety of crops, including olive trees, cotton and horticultural crops (Pegg and Brady, 2002). Because of its wide host range, long survival and vascular lifestyle, it is difficult to control. Isolates of *V. dahliae* exhibit high degrees of variability in the severity of symptoms induced in host plants. Moreover, the virulence of individual isolates may vary markedly on different hosts. Thus, specific pathotypes are likely to be important in particular crops. Isolates infecting olive or cotton can be classified into defoliating (D) or nondefoliating (ND) pathotypes, depending on their ability or otherwise to induce rapid defoliation of plants (Schnathorst and Mathre, 1966; Bejarano-Alcázar *et al.*, 1996). In countries such as Spain and Turkey, an alarming prevalence of the D pathotype in major olive-cultivation areas has been reported. This pathotype has been connected to the previous existence in the same areas of cotton crops infected by the pathotype (Rodríguez *et al.*, 2008; Dervis *et al.*, 2010; López-Escudero *et al.*, 2010; Jiménez-Díaz *et al.*, 2011; reviewed in Jiménez-Díaz *et al.*, 2012).

Corresponding author: M.D. García-Pedrajas
E-mail: mariola@eelm.csic.es

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It is unclear how the D pathotype initially appears in a particular geographical region. *V. dahliae* is an asexually-reproducing fungus, with exchange of genetic material thought to be restricted to isolates in the same vegetative compatibility group (VCG), defined as a group of isolates able to undergo hyphal anastomoses. Thus, different VCGs are thought to be genetically isolated populations (Rowe, 1995). All D isolates, regardless of their geographic origin, have been placed in VCG1A (Korolev *et al.*, 2001; Collado-Romero *et al.*, 2006; Dervis *et al.*, 2007; Korolev *et al.*, 2008; Dervis *et al.*, 2010; Jiménez-Díaz *et al.*, 2011). However, there is also genetic diversity among VCG1A/D isolates, and high variability in the virulence of individual D isolates on different hosts (Jiménez-Díaz *et al.*, 2012). Thus, the data available can fit two competing hypotheses on the appearance of the D isolates, which not necessarily exclude one another. The first hypothesis postulates that the D pathotype originated once and migrates worldwide, introduced with contaminated cotton seeds. The second hypothesis suggests that the pathotype has originated multiple times from native ND populations. For effective disease management, it is important to understand the sources and flow of the virulent D isolates, to focus on either assessing the potential of the pathogen for evolving new more virulent strains, or implementing measures to prevent exotic introductions.

Mycoviruses are widespread and have been detected and characterized in all major taxonomic groups of filamentous fungi (Ghabrial and Suzuki, 2009). Most known mycoviruses have double-stranded RNA (dsRNA) or single-stranded RNA genomes. They are unusual in the sense that they lack extracellular phases in their replication cycles, and are transmitted through intracellular mechanisms (Nuss, 2011). Therefore, they are not infectious in the classical sense, and can be considered intrinsic genetic elements of the fungal host, in which they frequently do not have marked effects. In phytopathogenic species, mycoviruses that induce hypovirulence, i.e. they reduce the virulence of their fungal hosts, have been described. They are of considerable interest because of their potential use as biological control agents (recently reviewed in Xie and Jiang, 2014). Plant pathogenic fungi are becoming an important source of new mycoviruses, identified in the search for those that induce hypovirulence, and their characterization is providing new insights into how mycoviruses interact with their fungal hosts (Dawe and Nuss, 2013). The growing number of mycovirus genomes available is also increasing our understanding of other aspects of viruses, including their taxonomy, ecology and evolution (Xie and Jiang, 2014).

Reports of the presence of mycoviruses in *V. dahliae* are still few. To date, only two viruses have been identified in this species, particularly in cotton isolates from China. These are, a chrysovirus, *Verticillium dahliae* chrysovirus 1 (VdCV1), found in an isolate collected in Saanxi Province (Central China) (Cao *et al.*, 2011), and a partitivirus, *Verticillium dahliae* partitivirus 1(VdPV1), identified in an isolate collected in Xinjiang Province (Northwestern China) (Feng *et al.*, 2013). Additionally, a novel partitivirus was recently identified in the related species *V. albo-atrum* (Cañizares *et al.*, 2014). In the present study, characterization of a new partitivirus strain present in an olive D isolate of *V. dahliae* from Turkey has provided data to support the long distance flow of *V. dahliae* isolates.

Materials and methods

Fungal material and growth conditions

We tested for the presence of mycovirus(es) in a collection of 16 *V. dahliae* isolates, of D and ND pathotypes, selected from the most important olive-producing areas of Turkey (Dervis *et al.*, 2010). Isolates, stored as conidial suspension in glycerol at -80ºC, were first grown on potato dextrose agar (PDA) plates at 24ºC for 4 d. Fragments of mycelium were then used to inoculate 100 mL of potato dextrose broth (PDB). Liquid cultures were incubated at 24ºC and 200 rpm for 4 d. Fungal material was then collected by filtration through filter paper, frozen with liquid nitrogen, and ground to a fine powder.

Isolation and molecular characterization of dsRNAs

Approximately 5 g of ground fungal tissue from each isolate was used for the extraction of dsRNAs using CF-11 cellulose (Morris and Dodds, 1979), and dsRNA molecules were fractioned on a 1% agarose gel. This analysis only provided evidence of viral infection in one of the isolates tested, D isolate Vd-253. Two dsRNA bands of approx. 1.7 and 1.5 kb were detected (Figure 1a) in this isolate. These bands were individually purified from the agarose gel, and sub-

jected to reverse transcription (RT) and PCR amplification using random hexamer priming to produce a cDNA library for each segment of what appeared to be a bi-partite virus. Analysis of these libraries generated a partial sequence of both segments. RT-PCR amplifications using pairs of sequence-specific primers were then used to produce cDNA clones to fill the sequence gaps of the regions not covered by the cDNA library clones. Clones from the terminal sequence of the dsRNAs were generated by T4 RNA ligase oligonucleotide-mediated amplification as described by Xie *et al.* (2006). The PCR primers used are listed in Supplementary Table 1. The full-length cDNA sequences for dsRNA1 and dsRNA2 were obtained by assembling the partial sequences in the different cDNA clones. All cDNA clones were sequenced in both orientations, and all sequences were confirmed using at least two independent overlapping clones. Sequencing was performed at the Genomic Unit of the University of Córdoba, Spain.

The sequences obtained were initially analyzed using the BLAST program of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and further analyzed at the nucleotide and amino acid levels using MEGA version 5 (Tamura *et al.*, 2011).

Results and discussion

Analysis of 16 *V. dahliae* isolates from Turskish olive tree for the presence of dsRNA viruses found evidence of viral infection only in one them, D isolate Vd-253 (Dervis *et al.*, 2010). This isolate was collected in Kahramanmaras Province of Turkey, and harbored two dsRNA segments (Figure 1a). Sequence analysis of these RNAs showed that the full-length dsRNA1 was 1767 base pairs (bp) in length, and putatively encoded a 539 amino acid (aa) protein highly similar to RNA-dependent RNA polymerases (RdRps) of partitivirus. The dsRNA2 was 1574 bp long and potentially encoded a 436 aa protein which showed similarity to coat proteins (CPs), also of partitivirus (Figure 1b). These sequences have been deposited in the GenBank database with accession numbers KJ700651 (RNA1) and KJ700652 (RNA2).

Comparison of the complete nucleotide (nt) and amino acid (aa) sequences among members of the partivirus family revealed that the partitivirus we identified in the Turkish D isolate Vd-253 and the partitivirus identified in the China isolate (VdPV1) (Feng *et al.*, 2013) are closely related. Sequence identities between these two micoviruses, at the nt level, were 94% for RNA1and 91% for RNA2, and 96% for RNA1 and 93% for RNA2 at the deduced aa sequence level. We therefore concluded that they are two strains of the same partitivirus. For this reason, we have designated the virus from the Turkish D pathotype isolate VdPV1-ol (VdPV1 from olive). As expected, phylogenetic analysis using RdRp and CP aa sequences of members of the family *Partitiviridae* placed VdPV1-ol in the same branch that VdPV1 (Figure 2a). The CP of the Chinese isolate, VdPV1, appears to be truncated, being shorter than that of related members of the genus *Gammapartitivirus*, in which VdPV1 is placed (Figure 2b). This is not the case in VdPV1-ol, since its CP ORF lacks the stop codon that gives rise to this early termination of translation, producing a CP 30 aa larger than that of VdPV1, and therefore more similar to the usual size in the related members of the genus *Gammapartitivirus*.

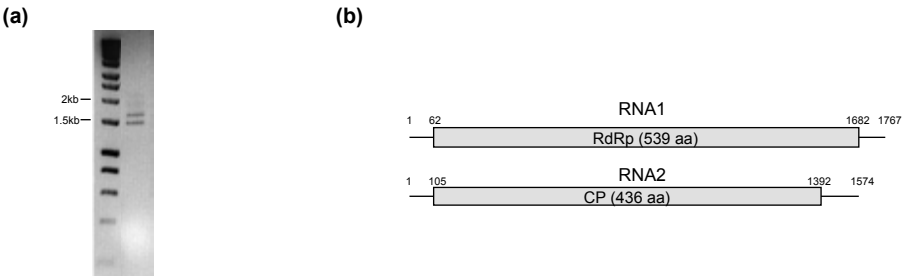

Figure 1. Characterization of *Verticillium dahliae* partitivirus 1 from olive (VdPV1-ol). (a) dsRNA banding pattern of *V. dahliae* D pathotype isolate Vd-253 from Turkey, analyzed by agarose gel electrophoresis. Size standard is shown on the left. (b) Schematic representation of the genomic organization of VdPV1-ol. The RdRp ORF (nt positions 62-1682 on RNA1) and the CP ORF (nt positions 105-1416 on RNA2) are represented by rectangular boxes.

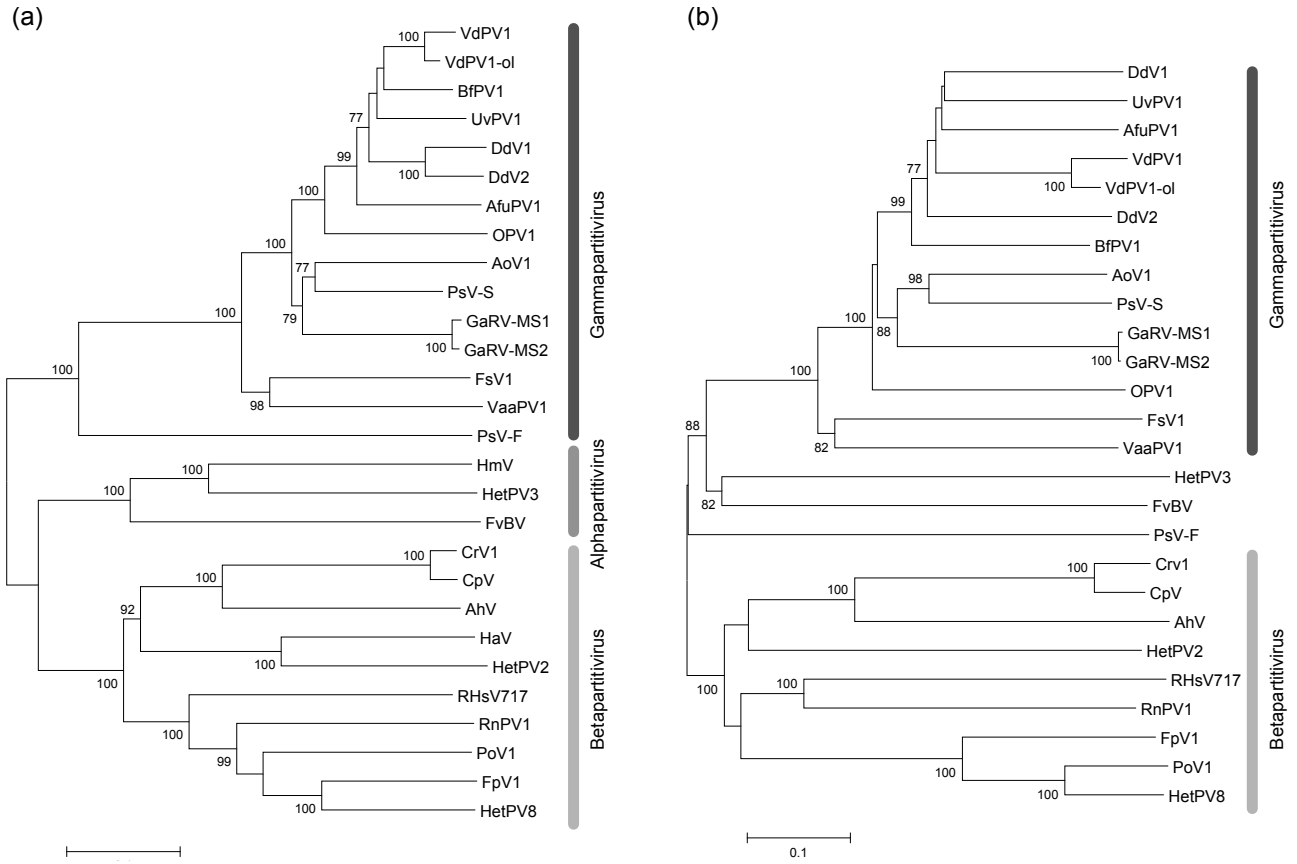

Figure 2. Phylogenetic analyses of VdPV1-ol and other members of the family *Partitiviridae*. (a) Phylogenetic tree based on the RdRp aa sequences. (b) Phylogenetic tree based on the CP aa sequences. Phylogenetic trees were constructed using the program MEGA 5.0, and generated by the neighbour-joining method. Bootstrap scores (1000 replicates) are shown at nodes when they are higher than 70%. Vertical bars on the right indicate coincidence with the recently reported grouping in partivirus genera. Sequences for the RdRp and CP were obtained from the GenBank database, and have the following accession numbers: *Aspergillus fumigatus* partitivirus 1 (AfPV1; FN376847, FN398100); *Aspergillus ochraceous* virus 1 (AoV1; EU118277, EU118278); *Atkinsonella hypoxylon* virus (AhV; L39125, L39126); *Botryotinia fuckeliana* partitivirus 1 (BfPV1; AM491609. AM491610); *Ceratocystis polonica* virus (CpV; AY260756, AY247205); *Ceratocystis resinifera* virus 1 (CrV1; AY603052, AY603051); *Discula destructiva* virus 1 (DdV1; NC_002797, NC_002800); *D. destructiva* virus 2 (DdV2; NC_003710, NC_003711); *Flammulina velutipes* browning virus (FvBv; AB465308, AB465309), *Fusarium poae* virus 1 (FpV1; AF047013, AF015924); *Fusarium solani* virus 1 (FsV1; D55668, D55669); *Gremmeniella abietina* virus MS1 (GaRV-MS1; NC_004018, NC_004019); *G. abietina* virus MS2 (GaRV-MS2; NC_006444, NC_006445); *Heterobasidium* partitivirus 2 (HetPV2; HM565953, HM565954), *Heterobasidium* partitivirus 3 (HetPV3; FJ816271, FJ816272); *Heterobasidium* partitivirus 8 (HetPV8; JX625227, JX625228.1); *Helicobasidium mompa* partitivirus (HmV; AB110979); *Heterobasidium annosum* partitivirus (HaPV; AF473549); *Ophiostoma* partitivirus 1(OPV1; AM087202, AM087203); *Penicillium sotoloniferum* virus F (PsV-F; AY7386, AY738337); *Penicillium stoloniferum* virus S (PsV-S; AY156521, AY156522); *Pleorotus ostreatus* virus 1 (PoV1; AY533038, AY533036); *Rhizoctonia solani* virus 717 (RHsV717; AF33290, AF133291); *Rosellinia necatrix* partitivirus 1 (RnPV1; AB113347, AB113348); *Ustilaginoidea virens* partitivirus 1 (UvPV1; KC503898, KC503899); *Verticillium albo-atrum* partitivirus 1 (VaaPV1; KJ476945; KJ476946); *V. dahliae* partitivirus 1 (VdPV1; KC422244.1; KC422243.1).

Comparison of the 5´- and 3´-untranslated regions (UTRs) of the dsRNA1 and dsRNA2 also highlighted the strong similarity of both *V. dahliae* partitiviruses; they were either identical (5´-UTRs of RNA1) or very similar (5´-UTRs of RNA2 and 3´-UTRs of both RNAs) in length, and essentially identical in

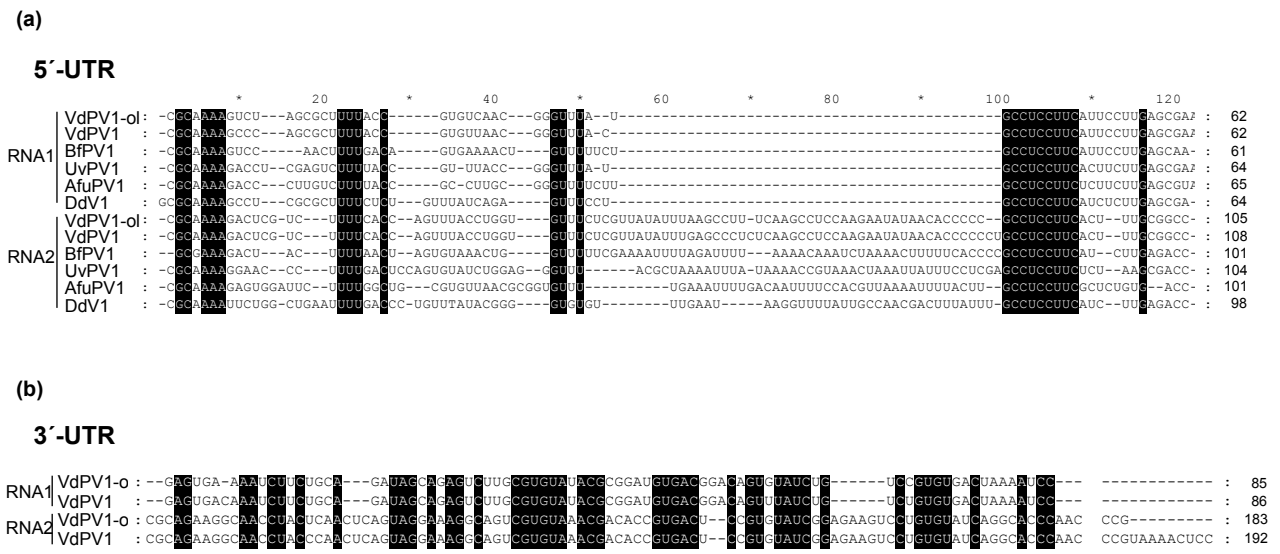

Figure 3. Comparisons of the 5´- and 3´-untranslated regions (UTRs) of the coding regions of RNA1 and RNA2 of VdPV1-ol. (a) Nucleotide sequence alignment of the 5´-UTR of the two genomic segments of VdPV1-ol and VdPV1 and the corresponding sequences from *Botryotinia fuckeliana* partitivirus 1 (BfPV1; AM491609.1, AM491610.1), *Ustilaginoidea virens* partitivirus 1 (UvPV1; KC503898.1, KC503899.1), *Aspergillus fumigatus* partitivirus 1 (AfPV1; FN376847.3, FN398100.2) and *Discula destructiva* virus 1 (DdV1; AF316992.1, AF316993.1). (b) Nucleotide sequence alignment of the 3´-UTR of the two genomic segments of VdPV1-ol and VdPV1. Black shading indicates identical nucleotides.

the nucleotide composition (Supplementary Figure 1). Multiple alignments of the 5´-UTRs of RNA 1 and RNA2 performed with other partitiviruses revealed the existence of shared identical stretches of nucleotides across the entire 5´-UTR sequence (Figure 3a). The conservation of these motifs between the 5´-UTRs of both RNA1 and RNA2 indicates that they may be involved in the formation of secondary structures to be recognized by the RdRp during the virus replication cycles. The 3´-UTRs of RNA1 and RNA2 of VdPV1-ol and VdPV1 also exhibited some stretches of sequence conservation (Figure 3b). However we did not identify similar motifs in other partitivirus members. These stretches of shared nucleotides in the 3´-UTRs could also be involved in the formation of secondary structures in the RNA recognized by the RdRp. We found that the 5´and 3´-UTRs of VdPV1-ol could potentially be folded into stable stem-loop structures (Supplementary Figure 2).

The nucleotide identity shared by the two partitivirus isolates identified in *V. dahliae* isolated from geographically distant areas (Turkey and China) suggests that they are related. Although no information is available about the VCG or pathotype of the cotton isolate (Feng *et al.*, 2013), it is probably a VCG1A/D isolate. Our results support the hypothesis that the presence of D pathotype isolates in a region can result from new introductions from distant geographical areas. The molecular differences indicate that VCG1A/D isolates from Spain and China could form a population distinct from the one formed by the VCG1A/D isolates from Turkey, Greece and Israel (Collado-Romero *et al.*, 2006; Korolev *et al.*, 2008). In contrast with previous molecular data, presence in them of the same viral species provides evidence of the connection between a Turkish D pathotype isolate and an isolate from China. The reason of this discrepancy could be that the D pathotype cotton isolates described in China in previous studies came mainly from the central regions of the country, while the Chinese isolate where the partitivirus was identified came from the Northwestern area, and there could be (at least two) different D pathotype populations in China. The isolation and molecular characterization of further mycoviruses from D pathotype populations from different geographical areas, together with the data provided by more classical approaches, could contribute to clear determination of the origins and spread of the D pathotype of *V. dahliae*.

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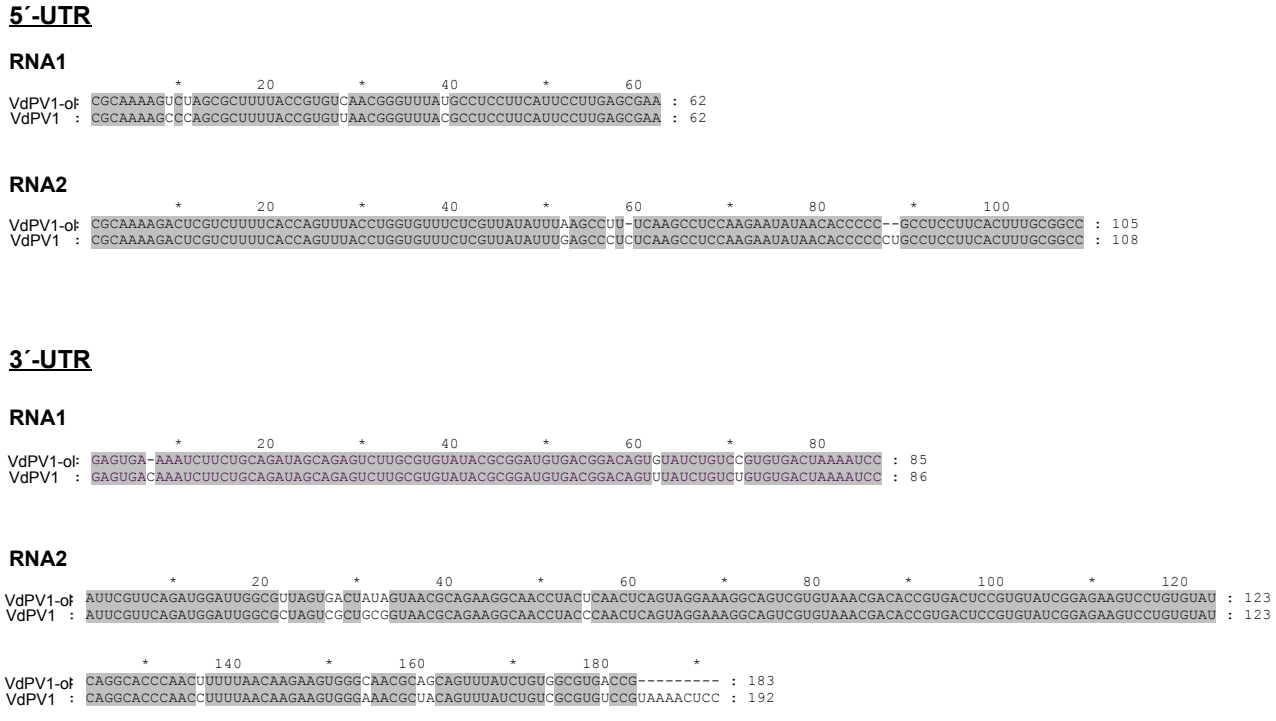
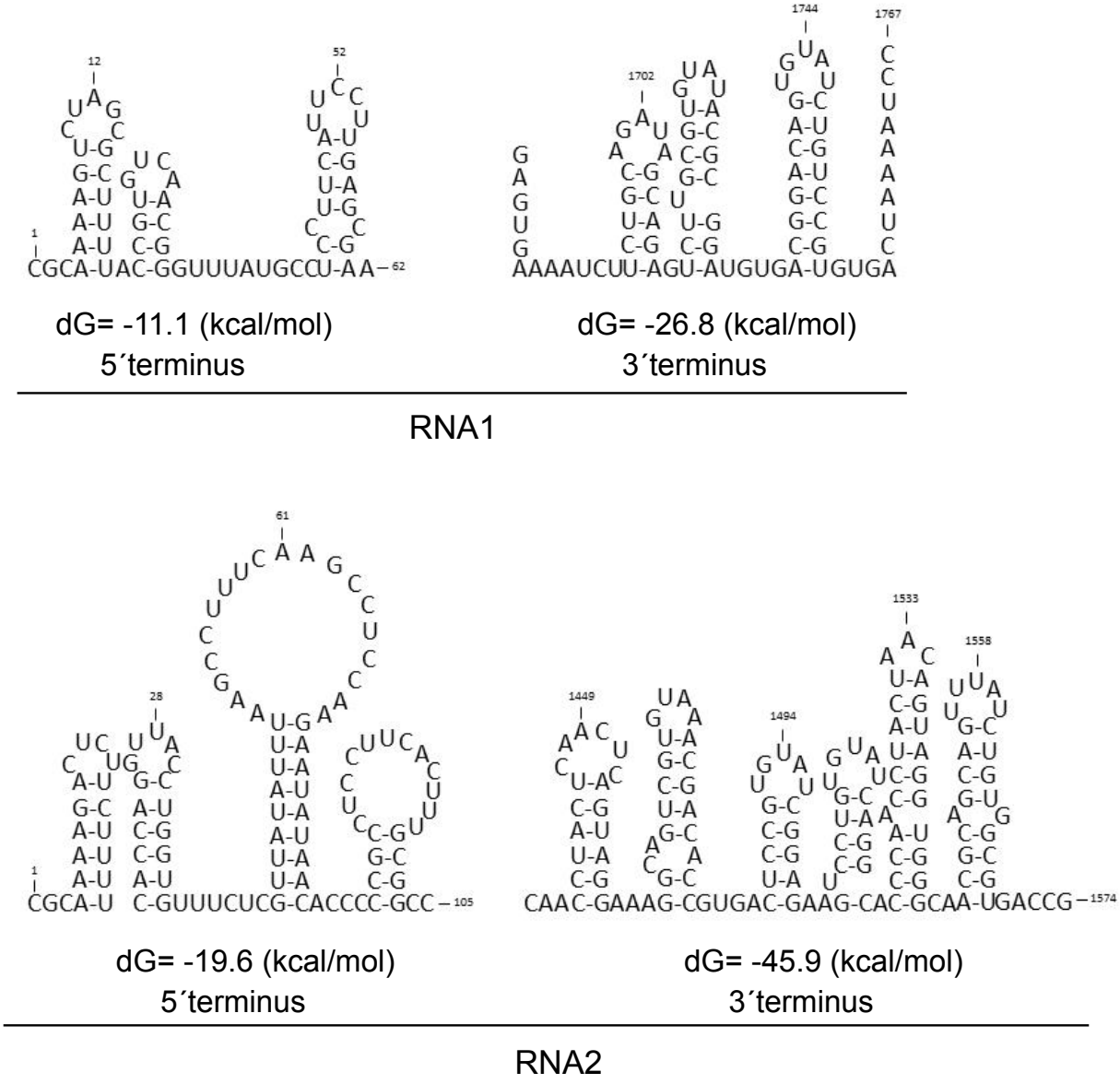
Supplementary Table 1. Primers designed in this study for the molecular characterization of VdPV1-ol

Name	Sequence (5`→3`)	Location	Polarity
Specific primers			
Vd1-R	CATCGAAGATTGCTGACTAG	dsRNA1: 434-415	-
Vd1-F	TCTGGTGACCAGTTTGATCT	dsRNA1: 1248-1267	+
Vd1C-F	GCTTGATTGGCATGAAGTGG	dsRNA1: 491-510	+
Vd1C-R	CATGAGGATTGGCGTGTTG	dsRNA1: 1217-1198	-
Vd2-R	TGCTTGACGTTCAGCCTTAC	dsRNA2: 207-188	-
Vd2-F	AAGTCTTATCGTGATGCTCC	dsRNA2: 970-989	+
Vd2C-F	CATGAGTTCACCAAGGATC	dsRNA2: 469-487	+
Vd2C-R	GGACGATCACGATAAGACTT	dsRNA2: 989-970	-
Adaptor and terminal primers			
Mod1 (3`-adaptor)	[Phos]GGTTCTACTCCTTCAGTCCATGTCAGTG TCCTCGTGC-(NH2)	to 3`-end of dsRNA	
End1	CACGAGGACACTGACATGG	complementary to the adaptor Mod1	
End2	ATGGACTGAGGAGTAGAAC	complementary to the adaptor Mod1	



Supplementary Figure 1. Sequence alignment of the 5´- and 3´-untranslated regions (UTRs) of the coding strands of RNA1 and RNA2 of VdPV1-ol and VdPV1. Gray shading is used to highlight conserved residues in the aligned sequences.



Supplementary Figure 2. Potential predicted secondary structures of the 5´and 3´UTRs of the coding strand of dsRNAs 1 and 2 of VdPV1-ol. The RNAs were folded and the lowest energies were estimated using RNAstructure software (Mathews, 2014).

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